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Differential induction of electrophile-responsive element-regulated genes by $n-3$ and $n-6$ polyunsaturated fatty acids

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Abstract In this study the $n-3$ polyunsaturated fatty acids (PUFAs) eicosapentaenoic acid and docosahexaenoic acid appear to be effective inducers of electrophile-responsive element (EpRE) regulated genes, whereas the $n-6$ PUFA arachidonic acid is not. These $n-3$ PUFAs need to be oxidized to induce EpRE-regulated gene expression, as the antioxidant vitamin E can partially inhibit the PUFA induced dose-dependent effect. Results were obtained using a reporter gene assay, real-time RT-PCR and enzyme activity assays. The induction of EpRE-regulated phase II genes by $n-3$ PUFAs may be a major pathway by which $n-3$ PUFAs, in contrast to $n-6$ PUFAs, are chemopreventive and anticarcinogenic.

Keywords: Chemoprevention; Electrophile-responsive element; Gene induction; Lipid peroxidation; Phase II detoxification; Polyunsaturated fatty acids

1. Introduction

Epidemiological and experimental studies have demonstrated that $n-3$ polyunsaturated fatty acids (PUFAs) reduce colon cancer risk [1–4]. However, the mechanisms by which $n-3$ PUFAs reduce cancer risk are still a matter of debate [5]. Several mechanisms for the chemopreventive characteristics of $n-3$ PUFAs have been postulated in the literature: metabolism of fatty acids to the biologically active prostaglandins, regulation of the peroxisome proliferator-activated receptor alpha, changes in membrane composition – which could affect cell signaling pathways –, and increase of oxidative stress [6–8]. In contrast to $n-3$ PUFAs, $n-6$ PUFAs seem to have no chemopreventive characteristics. Moreover, it has been reported that $n-6$ PUFAs promote intestinal tumorgenesis [9,10].

When regarding oxidative stress, the adaptation of cells to this stress is critical. Foremost is the transcriptional regulation of antioxidant enzymes, many of which are controlled by an electrophile-responsive element (EpRE) enhancer element, also known as an antioxidant-responsive element [11]. The major transcription factor involved in EpRE-mediated gene transcription regulation is NF-E2-related factor 2 (Nrf2) [12,13]. Under normal conditions, Nrf2 is sequestered by binding to the cysteine-rich Kelch-like ECH-associated protein 1 (Keap1). Inducers can react with the cysteine thiols of Keap1 leading to a conformational change and subsequent release of Nrf2, allowing interaction with EpRE elements [12–16]. One of these inducers is a member of the J-series cyclopentenone prostaglandins. These compounds are analogous to isoprostanes, which are formed during non-enzymatic lipid peroxidation of PUFAs, suggesting a possible relation between oxidation of PUFAs and EpRE induction [11,17].

Besides antioxidant enzyme genes, EpREs also regulate genes encoding phase II detoxification enzymes [13]. These phase II enzymes play an important role in determining the final fate of carcinogens/procarcinogens and their subsequent impact on carcinogenesis [18,19]. Therefore, the induction of phase II enzymes is considered a major process in cancer prevention [20].

To determine whether PUFAs can induce EpRE-mediated genes, the EpRE (mGST-Ya)-LUX cell line was used, which is stably transfected with an EpRE-controlled luciferase reporter gene [21]. This reporter cell line originates from the mouse hepatoma cell line Hepa-1c1c7, and makes use of an EpRE element from the mouse glutathione *S*-transferase-Ya EpRE gene regulatory region.

2. Materials and methods

2.1. Cultivation of the mouse EpRE(mGST-Ya)-LUX cell line

The transfected luciferase reporter cell line EpRE(mGST-Ya)-LUX was developed as described by Boerboom et al. [21]. The cells were cultivated in minimum essential medium α (MEM-α) medium, supplemented with (v/v): 10% fetal calf serum, 1% 250 μg ml^{-1} amphotericin B, and 0.1% 50 mg ml^{-1} gentamicin. The cells were maintained in an atmosphere of 5% CO_2/95% air at 37 °C with a humidity of 100%. After reaching 70–90% confluence in 75 cm^2 culture flasks, the cells were sub-cultured at a ratio of 1:5.

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Abbreviations: AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; EpRE, electrophile-responsive element; GST(P1), glutathione *S*-transferase (Pi 1); Keap1, Kelch-like ECH-associated protein 1; MT1, metallothionein 1; NQO1, NAD(P)H: quinone oxido-reductase 1; Nrf2, NF-E2-related factor 2; PUFA, polyunsaturated fatty acid

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2.2. Cell exposure

The trypsinized EpRE(mGST-Ya)-LUX cells were diluted with culture medium to a concentration of 2×10^5 cells ml^{-1}. After 24 h of growth, the cells were exposed to MEM-α medium containing: 1 mg ml^{-1} fatty acid-free bovine serum albumin (BSA), the indicated concentrations of the fatty acid to be tested, and, when applicable, 100 μM vitamin E. If applicable, the cells were pre-incubated with antioxidant vitamin E during the first 24 h of growth. The fatty acids were added to the medium as a solution in ethanol; the final concentration of ethanol in the medium of 0.5%. Before addition to the cells, the fatty acids were pre-incubated in the BSA-containing medium at 37 °C for 30 min. The positive control wells received exposure medium containing a final concentration of 40 μM *tert*-butyl hydroquinone (*t*BHQ) in ethanol instead of the fatty acid solution.

2.3. EpRE-LUX assay

The EpRE(mGST-Ya)-LUX cells were exposed in a Packard ViewPlate-96. After 24 h of exposure the plate was rinsed with 0.5× phosphate-buffer saline. Subsequently, the cells were lysed by adding 30 μl of a hypotonic low-salt buffer (10 mM Tris/HCl, pH 7.8, 2.0 mM 1,2-diaminocyclo-hexane-tetraacetic acid, and 2.0 mM dithiothreitol) per well. The plate was put on ice for 10 min, and frozen at −80 °C for 1 h. Before analysis and protected from light, the plate was thawed on ice for 30 min and subsequently, it was gently shaken to reach room temperature. Luminescence was analyzed using a Luminoskan (Thermo Labsystems, Altricham, UK) with the following protocol for each well: measurement of the background light emission for 2 s, addition of 100 μl flashmix (20 mM Tris/HCl, pH 7.8, 1.07 mM $MgCO_3$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 2 mM DTT, 470 μM D-luciferin, 5 mM ATP), measurement of the light emission for 2 s, and termination of the light reaction by addition of 50 μl of 0.2 M NaOH.

2.4. Real-time quantitative RT-PCR analysis

RNA was isolated from exposed EpRE(mGST-Ya)-LUX cell cultivations, grown in 25 cm^2 culture flasks, using Trizol according to the manufacturer's protocol (Invitrogen™), and purified using the Qiagen's RNeasy Mini Kit and RNase-Free DNase Set. First-strand cDNA synthesis was carried out with an oligo$(dT)_{15}$ primer and Moloney murine leukemia virus reverse transcriptase (Promega); during synthesis the recombinant ribonuclease inhibitor RNaseOUT™ (Invitrogen™) was present. The amplification reaction was carried out on a LightCycler (Roche Diagnostics) with gene-specific primers and used the SYBR Green 1 protocol. The following LightCycler protocol was used: 15 min heat start at 95 °C; 45 cycles of denaturation at 95 °C for 30 s, annealing at the optimal annealing temperature for the primer set for 30 s, and extension at 72 °C for 45 s; and a terminal extension at 72 °C for 5 min. Fluorescence detection was carried out at 72 °C. The Bio-Rad Gene Expression Macro ($\Delta\Delta C_T$) was used for analysis. Relative expression ratios were normalized to the housekeeping gene β-actin. The primer sequences are available on request.

2.5. NQO1 and GST activity assay

EpRE(mGST-Ya)-LUX cells were exposed in six-well tissue culture plates. After 24 h of exposure the cells were trypsinized and resuspended in MEM-α medium. This cell suspension was centrifuged for 5 min at $80 \times g$. Then, the supernatant was decanted and the cell pellet resuspended in 20 mM Tris/HCl, pH 7.5. The cells were lysed using a Bandelin Sonorex RK100 for 5 min. Again, the samples were centrifuged again for 5 min at $80 \times g$. The total amount of protein in the cell-free extracts was determined using the bicinchoninic acid assay (Pierce) with bovine serum albumin as a standard.

For the NAD(P)H:quinone oxido-reductase 1 (NQO1) measurements, an adaptation of the assay as described by Ernster [22] and modified by Benson et al. [23], was used with 2,6-dichlorophenolindophenol (DCPIP) as a substrate. The reaction mix contained: 20 mM Tris/HCl, pH 7.5, 0.01% Tween 20 (v/v), 0.07% BSA (w/v), 200 μM NADH, 2% cell-free extract (v/v), and 40 μM DCPIP; assays were carried out in the presence or absence of 20 μM dicumarol. NQO1 activity is described as the dicumarol-inhibitable decrease in absorbance at 600 nm with DCPIP as a substrate and is expressed in nanomoles of DCPIP reduced per minute per milligram of protein.

For the GST measurements, an adaptation of the assay as described by Habig et al. [24] was used with 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate. The reaction mix contained: 100 mM potassium phosphate, pH 6.5, 1 mM CDNB, 1 mM reduced glutathione and 2% cell-free extract (v/v). GST activity is described as the increase in absorbance at 340 nm with CDNB as a substrate and is expressed in nanomoles of CDNB–glutathione conjugate formed per minute per milligram of protein.

2.6. Statistics

Statistical significance was tested using a one-tailed homoscedastic Student's *t* test. The α level was set at 0.05.

3. Results and discussion

The EpRE(mGST-Ya)-LUX cell line was exposed to the $n-3$ PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), and the $n-6$ PUFA arachidonic acid (AA) in a concentration range of 0–100 μM for 24 h. EPA and DHA were selected as these are the most abundant $n-3$ PUFAs in the human diet. AA was chosen as an $n-6$ PUFA because it is also present in the human diet and resembles EPA and DHA the most with respect to fatty acid chain length and saturation. The concentrations used are within range of the normal PUFA concentration in blood plasma [25]. After exposure the luciferase activity induced in the reporter cells was measured; the results are expressed as an induction factor (Fig. 1A). To assess the necessity of PUFA oxidation for the induction of EpRE-regulated gene transcription, the antioxidant vitamin E was added at a concentration of 100 μM (Fig. 1B). *t*BHQ, a standard inducer of EpRE-regulated gene transcription, was used as a positive control [26].

The results presented in Fig. 1A show a dose-dependent increase in EpRE-controlled luciferase reporter gene expression induced by $n-3$ PUFAs. Exposure to the highest concentration resulted in a large increase in luciferase induction of almost 8-fold for DHA, and 4.5-fold for EPA. In contrast, exposure to the highest concentration of the $n-6$ PUFA AA gave only a small 2.1-fold increase in induction. DHA and EPA started to differ significantly from AA from 20 μM onwards: $P = 0.0073$ and $P = 0.026$, respectively. All together, the results for this cell line show that firstly, all the tested PUFAs are able to induce EpRE-mediated luciferase. Secondly, the $n-3$ PUFAs EPA and DHA induce EpRE-mediated luciferase in a much higher fold change than the $n-6$ PUFA AA.

When comparing Fig. 1B to A, it is obvious that the antioxidant vitamin E strongly decreases the luciferase induction factor for all tested PUFAs. Exposed to the highest concentration of PUFA in the presence of 100 μM of vitamin E, the inductions are 3.7-fold, 2.0-fold and 1.4-fold for DHA, EPA and AA, respectively. Statistics show that the induction factors for the $n-3$ PUFAs DHA and EPA in the absence of vitamin E, start to differ significantly from the induction factors in the presence of vitamin E: for DHA from 20 μM ($P = 0.010$) and for EPA from 40 μM ($P = 0.017$) onwards. For the AA-mediated induction vitamin E had no significant effect ($P \geqslant 0.15$). These results clearly show that oxidation plays a major role in the $n-3$ PUFAs' ability to induce EpRE-regulated gene expression.

Subsequently, the results of the reporter gene studies were confirmed by real-time PCR analysis of the effect of $n-3$ and $n-6$ PUFAs on the expression level of three known EpRE-regulated genes: glutathione *S*-transferase Pi 1 (GSTP1), NQO1, and metallothionein 1 (MT1) [27,28] (Fig. 2). In addition, total GST and NQO1 enzyme activity upon exposure to DHA, EPA,

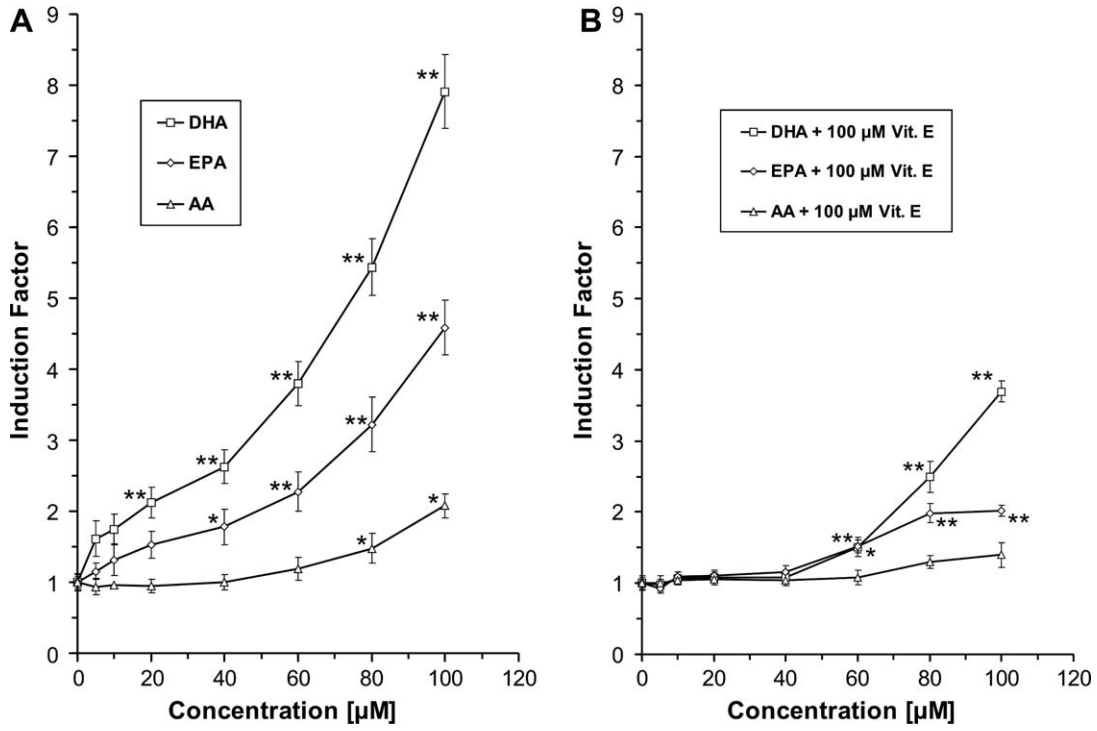

Fig. 1. Induction of luciferase in EpRE(mGST-Ya)-LUX cells after 24 h of exposure to increasing concentrations of DHA, EPA and AA, respectively ($n = 4$), expressed as the induction factor as compared to the solvent control, (A) in the absence of vitamin E and (B) in the presence of 100 μM vitamin E.

and AA were measured to determine the induction of the GST and NQO1 genes at the protein level (Fig. 3). For both assays the same cell line was used as for the luciferase assay.

Statistics show that using real-time RT-PCR (Fig. 2) the induction by DHA significantly differs from the control for all the three tested genes: GSTP1 ($P = 3.5E-05$), NQO1 ($P = 0.0041$), and MT1 ($P = 0.025$). Also the induction by EPA differs significantly from the control: GSTP1 ($P = 8.5E-05$), NQO1 ($P = 0.031$), and MT1 ($P = 6.5E-08$). AA only significantly ($P = 0.027$) differs from the control in the case of NQO1, but here there is repression instead of induction.

For the fold change of the enzymes (Fig. 3), only the $n-3$ PUFAs DHA and EPA significantly differ from the NQO1 control ($P = 2.3E-04$ and $P = 3.0E-05$, respectively). In the case of GST, all the tested PUFAs are inducers ($P = 2.0E-04$, $P = 1.9E-04$ and $P = 0.0037$ for DHA, EPA and AA, respectively). However, the induction by AA is significantly lower than those of the $n-3$ PUFAs DHA and EPA ($P = 4.05E-04$ and $P = 3.83E-04$, respectively).

When all the results that are shown in Figs. 2 and 3, are compared, we conclude that the differential induction by $n-3$ and $n-6$ PUFAs of EpRE-mediated genes is comparable to the results obtained in the reporter gene assay: DHA has the highest induction factor, followed by EPA, and then AA. For all EpRE-regulated responses, except for the GST activity, no induction by AA is observed.

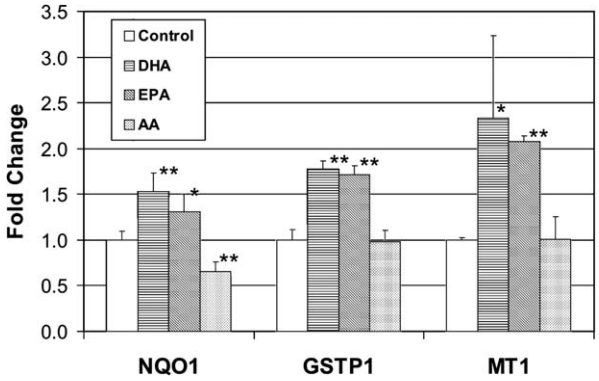

Fig. 2. Fold change, determined by real-time RT-PCR, of the NQO1, GSTP1 and MT1 mRNA levels in EpRE(mGST-Ya)-LUX cells after 24 h of exposure to 100 μM DHA, EPA and AA, respectively ($n = 4$), as compared to the solvent control.

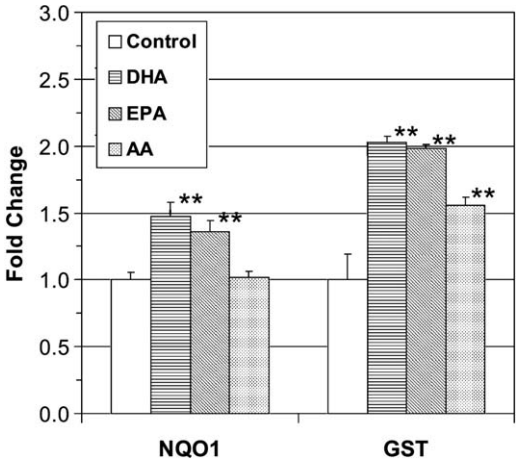

Fig. 3. Fold change of the enzyme activity of NQO1 and GST in EpRE(mGST-Ya)-LUX cells after 24 h of exposure to 80 μM DHA, EPA and AA, respectively ($n = 4$), as compared to the solvent control.

The induction observed in the reporter assay was higher than the induction of the endogenous genes studied, but there are several possible explanations that can account for this difference. The reporter assay was based on the EpRE element from the regulatory region of the mouse GST-Ya gene, and this element tends to mediate higher induction levels than the EpRE element controlling expression of the NQO1 gene [21]. Moreover, the expression of the endogenous GSTP1 and NQO1 genes is not only controlled by an EpRE element, as is the case with the luciferase reporter gene, but by a more complex regulatory region including additional regulatory sequences, which might explain these quantitative differences.

Our data on enzyme induction by $n-3$ PUFAs are in agreement with recent observations by Arab et al. [29]. Upon exposure of human fibroblasts to DHA they found a dose-dependant induction of mRNA and enzyme activities of γ-glutamyl cysteinyl ligase, glutathione reductase, and glutathione *S*-transferase, all involved in glutathione-homeostasis. On the basis of the presence of a consensus EpRE(ARE) sequence upstream of these genes they suggested a role for antioxidant-responsive elements EpRE(ARE)-mediated transcription regulation of the antioxidant response in fibroblasts when exposed to DHA. In our reporter gene study we provide conclusive evidence for the EpRE(ARE)-mediated nature of the antioxidant enzyme induction by the $n-3$ PUFA DHA, and moreover, for the first time show that this is a clear and biological relevant differential effect as compared to $n-6$ PUFAs.

Altogether, we conclude that $n-3$ PUFAs are effective inducers of EpRE-regulated genes, while $n-6$ PUFAs are not. The mechanism by which $n-3$ PUFAs can protect against cancer might be the induction of EpRE-mediated phase II detoxification enzymes, thereby increasing the defense capacity of the cells towards potential carcinogens [13,20]. The presented results demonstrate that the induction of EpRE-mediated cancer-protective gene expression by $n-3$ PUFAs may be a major pathway by which $n-3$ PUFAs are chemopreventive and anticarcinogenic.

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